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Optimizing packing of live seahorses for shipping

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ABSTRACT

The packing and shipping of live marine organisms always poses a potential risk to their survival and well-being, with the costs associated with these practices being paramount for marine ornamental species value chains. The present study describes two experiments employing the longsnout seahorse *Hippocampus reidi* (~80 mm) as a model seahorse species to optimize its packing methods for live shipping. The first experiment evaluated the combined effect of seahorse density (1 ind. per 300 mL, 1 ind. per 450 mL and 1 ind. per 600 mL), transit time (24 vs. 48 h), and use of an oxygen saturated atmosphere for packing (pure oxygen vs. compressed air). The second one evaluated the combined effect of water salinity (15, 25, and 35) and use of a substrate for packed specimens to hold onto it, at a density of 1 ind. per 300 mL. Survival was 100% in all treatments in both experiments up to 48 h after shipping, with ~90% of seahorses displaying a normal feeding behaviour immediately post-transportation. In the first experiment, no significant differences were found ($P > 0.05$) in weight-specific total ammonia nitrogen (TAN) excreted in all treatments within the same transit time. At the end of the transit time, treatments with an oxygen saturated atmosphere displayed an oversaturation in dissolved oxygen (DO) concentrations, whereas those employing compressed air for 48 h ended the experimental trial with a DO concentration above 80%. Water with a salinity of 15 promoted a significantly ($P < 0.05$) lower decrease in pH, followed by water at a salinity of 25 and 35. The lower salinity employed may have reduced breathing frequency of seahorses during transport. The presence of a substrate significantly ($P < 0.05$) decreased weight-specific TAN excreted, possibly due to stress reduction. Overall, *H. reidi* can be packed at a density as low as 1 ind. per 300 mL for up to 48 h, with the use of pure oxygen not being mandatory. Lower salinities and the use of substrate can enhance seahorse welfare when these are shipped over longer transit times without representing additional significant costs. Overall, the findings of the present study may allow traders to ship 3 times more live seahorses than they currently do without negatively impacting their welfare neither increasing associated shipping costs.

1. Introduction

Thousands of seahorses are traded live every year as marine ornamental species to supply the aquarium industry (Foster et al., 2016). As most marine ornamental species, seahorse trade involves transporting live specimens for long-distances by airplanes. Therefore, packing and shipping represent a major cost for traders and pose high mortality risks for the animal (Lim et al., 2007; Wabnitz et al., 2003; Wood, 2001), making the transport a key step for value and production chains. While remarkable improvements have been reported in seahorse aquaculture (Olivotto et al., 2011), researchers and enterprises have mostly overlooked the development of innovative shipping strategies when compared with other culture-related topics. To our best knowledge, there is no multifactor experimental-based study available on packing methods for live seahorses. Thus, improving transport methods for these highly priced organisms may bring economic benefits for producers and secure animal welfare (Cohen et al., 2017).

Most aquatic ornamental species are shipped in closed plastic bags filled with one third of water and two-thirds of pure oxygen inside Styrofoam boxes (reviewed in Correia and Rodrigues, 2017). The size of the bag and the volume of water being used depends on fish species, size and density. Marine ornamental species are pricey products and

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thus, it is a common practice to pack them at a low density (Cole et al., 1999; Lim et al., 2007), commonly a single specimen per bag (mono-packing). Nonetheless, one should consider that the weight of the water being shipped always surpasses that of the fish itself and thus, transportation cost is highly dependent on the amount of water being used per specimen during shipping.

Presently, there is no consistency on packing methods for live seahorses in the industry. Major traders of seahorses employ from mono-packing of *H. reidi*, at densities as low as 1 specimen per L, up to 5 specimens per 2 L (1 ind. per 400 mL) (Miquel Planas and Maik S.C. Hora, person. comm., 2017). Nonetheless, the unique morphology, biology and physiology of seahorses suggests that they can be packed in lower water volumes than other reef fish and without requiring the use of pure oxygen. In other words, it appears to exist room to optimize and standardize packing methods. For example, seahorse upright body position may allow traders to use a narrower bag for packing, thus decreasing the water volume used per specimen. Seahorses are ambush predators, have a low swimming capacity and remain attached to a substrate for long periods of time (Foster and Vincent, 2004). These features suggest a low metabolism and possible resilience to stress caused by transportation. Indeed, an experiment with *Hippocampus abdominalis* showed that this species has a fast recovery after transportation stress (Wright et al., 2007), corroborating the hypotheses above.

To ship live fish successfully, one must ensure suitable oxygen concentrations and water quality. Transit time may vary from < 24 h in domestic transportation up to 72 h on an international shipping (commonly averaging 48 h) (Cole et al., 1999). During shipping, fish consume oxygen, release CO_2 and excrete ammonia to the holding water. Air is more pressurized in closed bags than in the atmosphere and hence oxygen present in the shipping bag rapidly dissolves into the water as packed fish consume it, often not being a limiting factor (Berka, 1986). Nonetheless, the accumulation of CO_2 in closed shipping bags decreases water pH and may affect oxygen-carrying capacity of hemoglobin, regardless of oxygen availability (Berka, 1986; Lim et al., 2007). Notably, under low pH conditions (e.g., < 7.5), most ammonia nitrogen present in marine water is in the form of the ammonium ion (NH_4^+), which is less toxic to fish than un-ionized (NH_3) (Boyd, 2015). Yet, excreted ammonia can reach toxic levels depending on fish species, size, density, stress condition and transit time. A common practice employed to reduce fish excretion and oxygen demand during transportation is fasting the fish for a few days prior to packing.

Water quality on shipping is also closely related to fish stress (Portz et al., 2006). Stressed fish excrete more ammonia, are more sensitive to ammonia toxicity, may display osmoregulatory dysfunction and are more likely to consume higher levels of oxygen (Carneiro and Urbinati, 2001; Randall and Tsui, 2002). Thus, by ensuring optimal packing conditions that minimize fish stress during shipping, one may significantly improve the chances of successfully transporting live specimens. The salinity of shipping water may play an important role in controlling stress and oxygen consumption during transport. Adding salts to freshwater is a common practice to control osmoregulation dysfunctions, or other physiologic disorder, caused by stress when transporting live freshwater fish (Carneiro and Urbinati, 2001; Lim et al., 2007; Long et al., 1977; McDonald and Milligan, 1997). Therefore, a similar rationale may also be valid for saltwater fish, if one considers a decrease in salinity of shipping water. Another issue that remains unaddressed for seahorses is the relevance of adding a substrate to which they may hold. By adding a substrate during transport, it is likely that live seahorses may present lower energy expenditure to stabilize their position in the shipping water, consequently being less prone to stress.

This work evaluated the effect of packing density, transit time, use of an oxygen saturated atmosphere, water salinity and the presence of a substrate to which seahorses may hold on the shipping of *Hippocampus reidi*, one of the most heavily traded seahorse species in the marine aquarium industry (Foster et al., 2016; Cohen et al., 2017). We considered both animal welfare and possible benefits for diminishing shipping costs of traded specimens to evaluate the optimal packing procedure for live seahorses.

2. Material and methods

Two experiments were performed to test the effect of five factors relevant for the shipping of live seahorses. In the first one, it was tested the effect of density, transit time (the period from packing to unpacking) and the use of an oxygen saturated atmosphere. In the second experiment, it was tested the effect of salinity and the use of substrate where seahorse could hold during transportation.

In both experiments, longsnout seahorses (*Hippocampus reidi*) with ~80 mm of total length (TL) were used as a representative model for live trade of seahorses for marine aquariums; all specimens were bred in captivity in the facilities of Instituto de Investigaciones Marinas (CSIC) in Vigo (Spain) (for detailed description of cultured methods for this species, please refer to Planas et al., 2017). This study complied with the bioethical requirements from the Regional Government (Consellería do Medio Rural de Pontevedra, Spain #ES360570202001/16/EDU-FOR07/MPO0) and CSIC bioethics committee.

Seahorses were kept for 24 h without food before packing. After packing, bags with seahorses were placed inside closed Styrofoam boxes. These boxes were kept in the laboratory under controlled room temperature (~21 °C) and gently agitated for approximately 5 min every hour, except during 12 consecutive hours at night. This movement aimed to mimic real shipping conditions experienced by live seahorses.

2.1. Experiment 1: density, transit time and oxygen saturated atmosphere

The effect of density, transit time and oxygen saturated atmosphere were tested through a factorial experiment in a randomized block design with five replicates per treatment. Density was tested as the volume of water employed to pack a single specimen (mono-packing): 300 mL, 450 mL and 600 mL. Transit times tested were 24 h and 48 h. To evaluate the necessity of an oxygen saturated atmosphere inside shipping bags, it was tested the use of pure oxygen contrasted with compressed air. The ratio water:air inside the shipping bags was standardized at 1:2. Thus, to respect a minimum water column of ~85 mm to cover the seahorse, it were used three different shaped bags for this experiment (Fig. 1).

Overall, 60 seahorses were divided in five blocks with 12 treatments each – one Styrofoam box was employed per block. The water used for packing seahorses was 1 μm filtered UV-irradiated seawater retrieved from the grow-out system where all seahorses were stocked prior to the experiment.

2.2. Experiment 2: salinity and substrate

The effect of salinity and substrate were tested through a factorial experiment in a completely randomized design with five replicates per treatment. Based on preliminary results from experiment 1, each specimen employed was packed in a bag with 300 mL of water and 600 mL of pure oxygen for 48 h. Salinities tested were 15, 25 and 35. These salinities were selected considering that *H. reidi* has an isosmotic point of ~12 (Hora et al., 2016) and that it has been commonly cultured in salinities between 24 and 35 (reviewed in Planas et al., 2017).

As seahorses were initially stocked in a system at a salinity of 35 before the experiment, specimens were acclimated to the salinities of 15 and 25 one day before packing. For this acclimation seahorses were randomly separated in three aquariums (~30 L each) in a static system with aeration and controlled temperature (25 °C) and freshwater purified through reverse osmosis was slowly dripped until the aquarium water matched the target salinities. This process took approximately 8 h

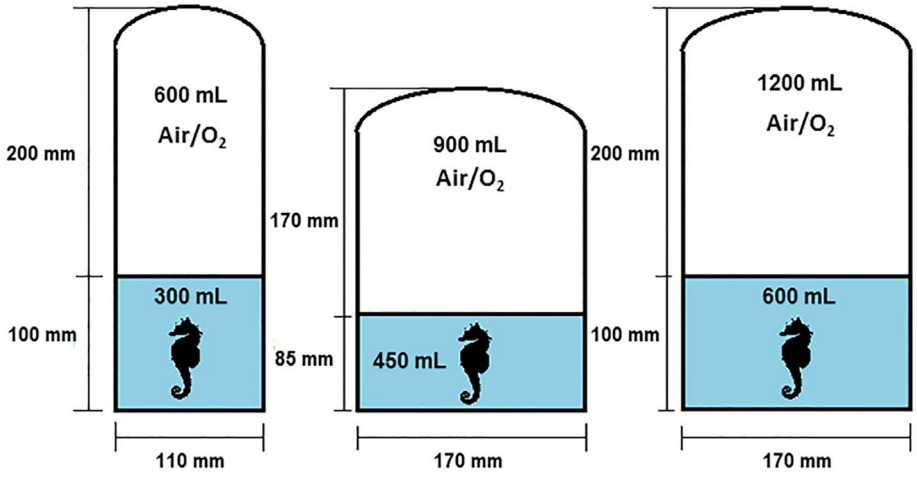

Fig. 1. Schematic representation of the three densities (number of seahorse per water volume) tested in the shipping experiment of live *Hippocampus reidi*.

and seahorses stayed in this system until they were packed in the next morning (~14 h after being acclimated). No seahorse died or displayed any distress signal during this acclimation process (e.g. Lying on the bottom, agitated swimming, high opercular beat frequencies, gasping up at the surface). In this experiment, the water used for packing was prepared by mixing 1 μm filtered UV-irradiated seawater with freshwater purified through reverse osmosis to match desired salinities when required and at the same temperature of the grow-out systems (~25 °C).

To evaluate the relevance of using a substrate, to which live seahorses can hold during shipping, half of the specimens were packed with a knot made of ~200 mm of hose (~5 mm diameter and ~5 g of weight) (Fig. 2). All specimens employed in this experiment were placed inside the same Styrofoam box.

2.3. Data sampling

Survival, total length and wet weight (WW) of each seahorse was evaluated 48 h post-transportation simulation, along with its feeding behaviour. After unpacking, seahorses were measured from the top of the coronet to the tip of the stretched tail with a ruler to the nearest 1 mm and weighted using a scale MXX-212 Denver Instrument® (Max. 210 g/d = 0.01 g). Seahorses size and weight were measured to verify if, by chance, larger or smaller animals could have been more allocated to a given treatment and act as a potential bias for experimental results. There were no significant differences ($P > 0.05$) on size and weight among treatments for both experiments, based on tree-way ANOVA (experiment 1) and two-way ANOVA (experiment 2) tests (see data analysis section for details). In experiment 1, average (± SD) TL was 81.3 ± 13.5 mm and average (± SD) WW was 1.68 ± 0.73 g (SD). In experiment 2, the average TL (± SD) was 85 ± 6.7 mm (SD) and average (± SD) WW was 1.79 ± 0.38 g.

To record survival 48 h post-transportation and monitor feeding behaviour, all seahorses were individually tagged with a coloured pendant in a nylon necklace, setting one colour code for each treatment (Fig. 3), and acclimatized to a new stocking system. This acclimatization consisted of pooling seahorses from same treatments (and their remaining transport water) in 3 L beakers (one per each treatment) and slowly dripping water from the new system until temperature and pH equalized. This process took approximately 1 h for both experiments. In this new system, two treatments were set (each with 10 seahorses) per aquarium (~30 L each), in a total of six aquariums for experiment 1

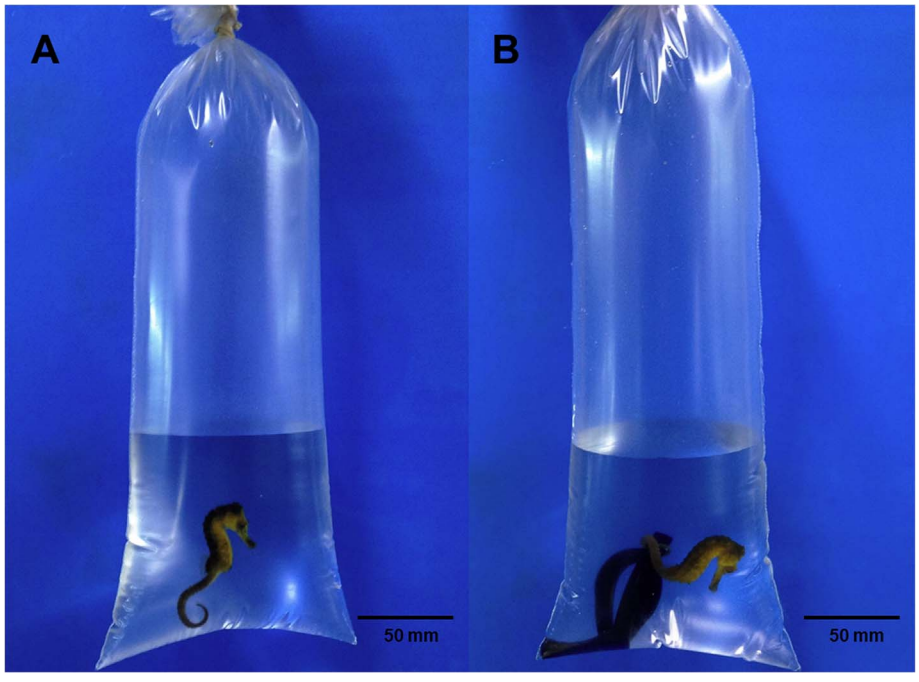

Fig. 2. Photo of a shipping experiment with seahorses (*Hippocampus reidi*) in bags with 300 mL water and 600 mL under an oxygen saturated atmosphere (pure oxygen). A: treatment without a substrate for the seahorse to hold; B: treatment with a substrate.

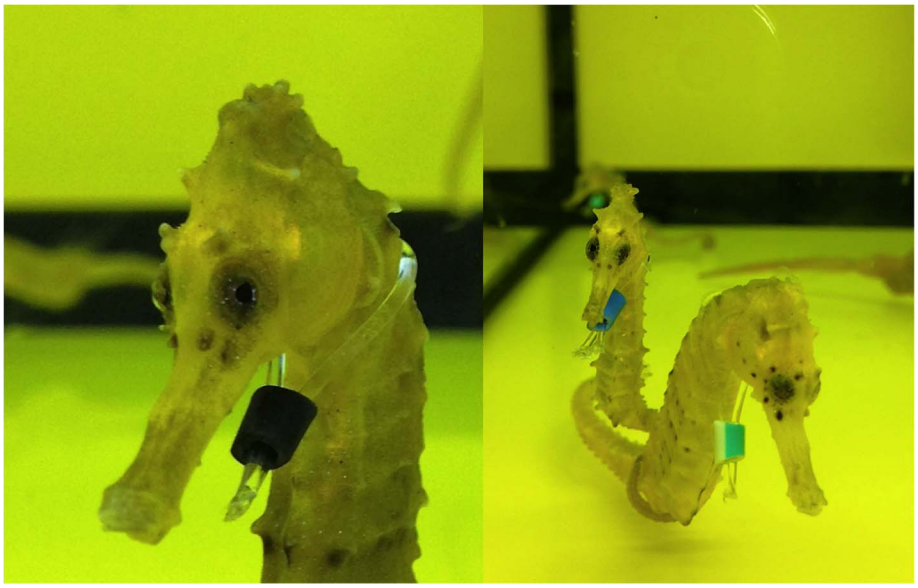

Fig. 3. Seahorses *Hippocampus reidi* with coloured pendant in a nylon necklace used to discriminate specimens from different treatments.

and three aquariums for experiment 2. For experiment 1, these aquariums were connected to a recirculated system, all sharing the same water for 48 h. As different salinities were tested in experiment 2, a static system was employed (no water sharing nor recirculation) and each aquarium set with a salinity of 15, 25 or 35. Seahorses remained in this system for 24 h with aeration, temperature control (25 °C) and partial water changes. After this period, these aquariums were also connected to a recirculated system by slowly dripping water until all aquariums reached a salinity of 35. Survival was then monitored for more 24 h with all treatments sharing the same water. In both experiments, live food (adult *Artemia* and metanauplii) was offered immediately after seahorses were introduced in the new system and monitored their feeding behaviour for 20 min. All specimens that did not ingest any live food during this observation period were considered as more stressed.

Water was analysed following the different steps of the processes, i.e., the initial water (packing water), water from the bags after transport (shipping water), and water from the systems where seahorses were placed after transport (new system water). It was measured the temperature, dissolved oxygen (DO), pH and total ammonia nitrogen (TAN). Temperature and DO were measured using a probe HI 98194 Multiparameter – Hanna®, whereas pH was measured with a pH meter micropH 2001 – Crison® (probe Hack 5208). Total ammonia nitrogen was determined following standard methods described in Limnologisk Metodik (1992). The packing and stocking system water were analysed for reference and control, with preliminary analysis revealing their optimal condition to perform both experiments (Table 1).

Table 1
Water parameters from initial water used for packing seahorses (*Hippocampus reidi*), and water from the systems where seahorses were placed after transport (new system water). TAN: Total ammonia nitrogen; DO: Dissolved Oxygen, S15, S25 and S35: treatments with salinity 15 25; and 35.

	Packing water				New system water			
	Exp. 1	Exp. 2			Exp. 1	Exp. 2		
		S15	S25	S35		S15	S25	S35
Salinity	38	15	25	35	35	15	25	35
pH	7.82	7.94	8.23	8.41	7.99	7.77	7.92	8.00
Temperature	25.5	24.7	24.6	25.2	24.8	24.6	24.4	24.9
DO ($mg{\cdot}L^{-1}$)	6.40	7.60	6.98	6.71	6.30	7.44	7.09	6.61
DO (%)	95.0	99.4	96.8	98.3	95.5	99.3	99.9	100
TAN ($\mu g{\cdot}L^{-1}$)	20.9	10.5	8.0	26.7	4.2	56.3	59.6	98.8
Nitrite[a] ($mg{\cdot}L^{-1}$)	0	0	0	0	0	0	0	0
Nitrate[a] ($mg{\cdot}L^{-1}$)	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5

[a] Determined with a colorimetric test for marine aquaria.

2.4. Data analysis

It was tested the decrease in pH (difference between pH before and after transport), weight-specific TAN excreted (total excreted ammonia divided by individual weight) and final DO concentration (DO after transport). For experiment 1, decrease in pH per hour was also tested (decrease in pH divided by the transit time), and weight-specific TAN excreted per hour. The water in all treatments employing an oxygen saturated atmosphere was still oversaturated in DO even after 48 h post-transportation; as these values were above the standard curve of the probe employed in the present study, these values are reported just as reference to the reader and were not used for any statistical analysis.

For experiment 1, a three-way ANOVA was employed to test interaction among the three independent factors tested (density, transit time and oxygen saturated atmosphere). As no significant interaction was recorded ($P > 0.05$), it was further applied the analysis by two-way ANOVA for factors density vs. oxygen saturated atmosphere (3 × 2) within each transit time using the variables decrease in pH and weight-specific TAN excreted. For final DO concentration test (only in treatments with compressed air), it was applied two-way ANOVA for factors density vs. transit time (3 × 2) because the variability between treatments with oxygen and compressed air were higher than between transit times. For experiment 2, it was applied a two-way ANOVA to evaluate the effect of both independent factors (salinity and substrate). For both experiments, when significant differences were recorded, the Tukey HSD was used for post-hoc comparisons. Shapiro-Wilk test and F-test were used to verify that the group of data fulfilled ANOVA assumptions of normality and homoscedasticity, respectively. The level of significance considered was 95%. Using values of final pH, temperature, salinity and TAN, it was estimated the concentration of un-ionized ammonia (NH_3) present in the bags after shipping based on the methods described by Khoo et al. (1977), modified from Whitfield (1974).

3. Results

3.1. Experiment 1: density, transit time and oxygen saturated atmosphere

Survival was 100% up to 48 h post-transportation. Except for two specimens in each of the three tested densities using compressed air and

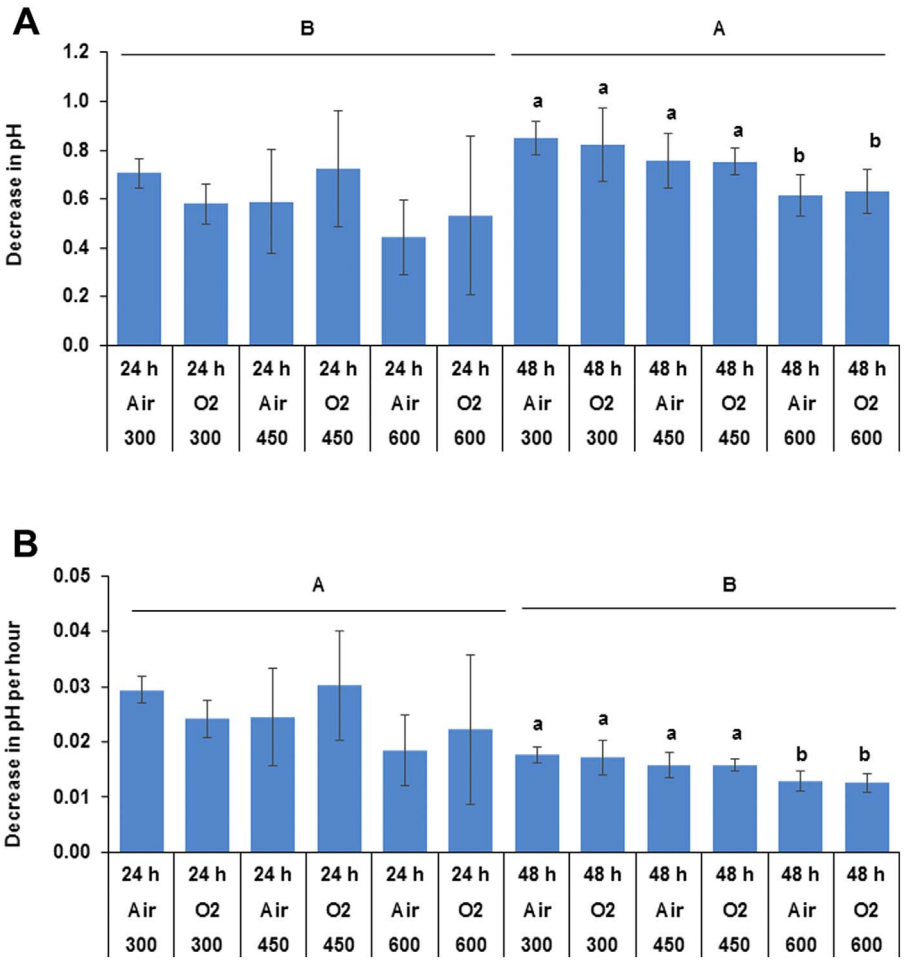

Fig. 4. Decrease in pH (A) and decrease in pH per hour (B) in shipping water used to transport live seahorses (*Hippocampus reidi*). Values are averages of five replicates and lines with upper and lower limits represent standard deviations for each treatment. Treatments are: 1 specimen per 300 mL (300), 450 mL (450), or 600 mL (600); bags with an oxygen saturated atmosphere (O_2) or compressed air (Air); and transit times of 24 h or 48 h. Letters indicate significant differences ($P < 0.05$) among treatments. No significant difference ($P > 0.05$) was found among treatments within a transit time of 24 h.

shipped for 48 h, all seahorses ate within < 10 min after acclimatization. Seahorses in these treatments were visually more agitated.

The average temperature (± SD) of shipping water was 21.1 ± 0.2 °C. The decrease in water pH and the concentrations of weight-specific TAN excreted in treatments simulating a transit time of 48 h were significantly ($P < 0.05$) higher than those recorded after 24 h (Figs. 4A and 5A). Nonetheless, when standardized per hour, both the decrease in pH and weight-specific TAN excreted in treatments for 48 h were significantly ($P < 0.05$) lower than after 24 h (Figs. 4B and 5B). The treatment employing the lowest density (1 ind. per 600 mL) displayed a significantly ($P < 0.05$) lower decrease in pH than the other two after 48 h (Fig. 4). Final average (± SD) pH values were 7.39 ± 0.21 (Min. 6.87) for treatments after 24 h, 7.37 ± 0.08 (Min. 7.25) for the lowest density tested (1 ind. per 600 mL) after 48 h and 7.20 ± 0.10 (Min. 6.93) for the other two packing densities also after 48 h. No significant difference was recorded ($P > 0.05$) in weight-specific TAN excreted among treatments within the same transit time (Fig. 5). The average (± SD) concentration of total estimated unionized ammonia was 0.001 ± 0.001 mg·L^{-1} (Max. 0.002 mg·L^{-1}) for treatments on each transit time (24 and 48 h). Treatments employing an oxygen saturated atmosphere ended transportation still displaying their water over saturated in DO, whereas all treatments employing compressed air displayed a level of DO saturation above 80% (Fig. 6). For reference, considering an atmospheric pressure of 1 atm – closed shipping bags have higher pressure – with a temperature of 21 °C and salinity 38, the DO saturation is ~7 mg·L^{-1}. Bags with the highest shipping density (1 ind. per 300 mL) that were filled with compressed air had significantly ($P < 0.05$) lower DO concentrations after transport (Fig. 6), regardless the transit time, with an average (± SD) of 6.0 ± 0.2 ppm.

3.2. Experiment 2: salinity and substrate

All shipped specimens survived up to 48 h post-transportation. All seahorses ate within < 5 min after acclimatization. The average temperature (± SD) of shipping water was 21.0 ± 0.0 °C. Water with a salinity of 15 displayed a significantly ($P < 0.05$) lower decrease in pH, followed by salinity 25 and 35, respectively (Fig. 7A). Final average (± SD) pH values were 6.95 ± 0.04 (Min. 6.87) for salinity 15, 7.06 ± 0.06 (Min. 6.96) for salinity 25, and 7.13 ± 0.06 (Min. 7.03) for salinity 35. The presence of a substrate to which seahorses could hold during transportation significantly decreased weight-specific TAN excreted (Fig. 7B). The average concentration of estimated unionized ammonia (± SD) was 0.004 ± 0.001 mg·L^{-1} (Max. 0.005 mg·L^{-1}) in treatments with a substrate and 0.005 ± 0.001 mg·L^{-1} (Max. 0.007 mg·L^{-1}) in treatments without a substrate. All treatments displayed over saturated DO after transportation.

4. Discussion

The survival of all seahorse specimens in both experiments after transport simulation and recovery, with most of them feeding normally immediately after transportation, suggests that *H. reidi* is remarkably hardy to shipping under different stocking densities, transit times, DO concentrations and water salinity. The ability of these fish to cope with salinity shifts was also evidenced in the present study because there was no mortality when salinity dropped from 35 to 15 in an 8-h period. This tolerance to lower water salinities is likely to be a common feature to all

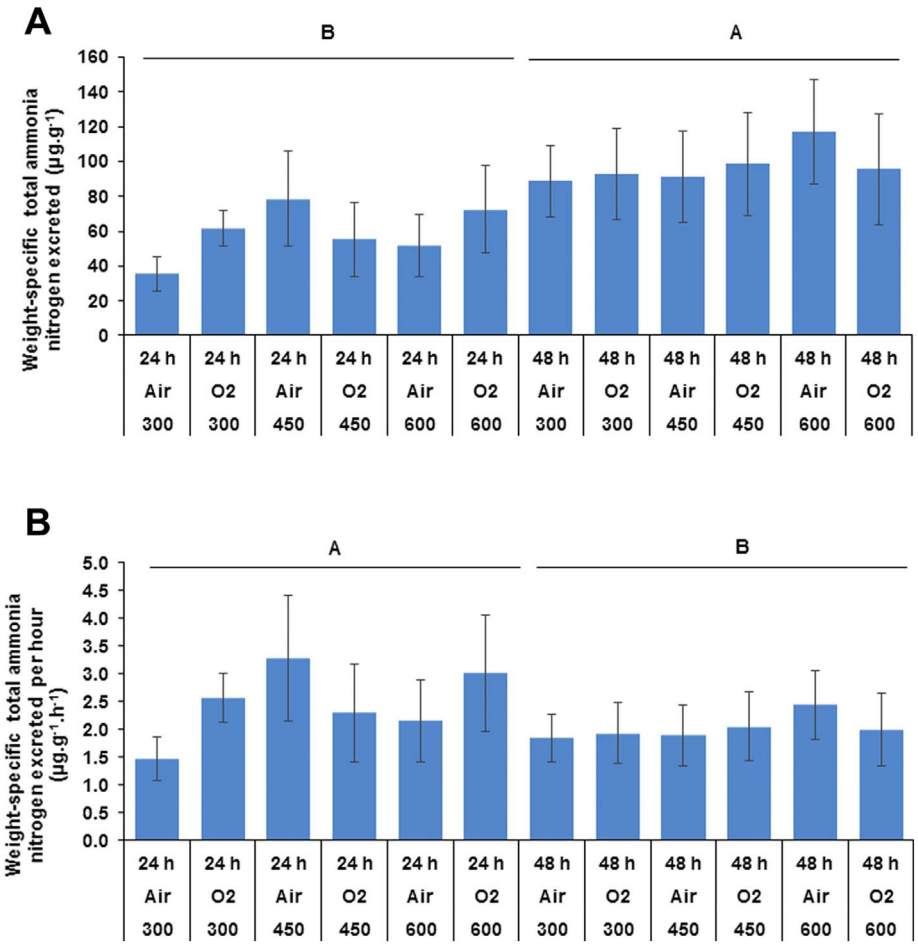

Fig. 5. Weight-specific total ammonia nitrogen excreted (A), and weight-specific total ammonia nitrogen per hour (B) in shipping water used to transport live seahorses (*Hippocampus reidi*). Values are averages of five replicates and lines with upper and lower limits represent standard deviations for each treatment. Treatments are: 1 specimen per 300 mL (300), 450 mL (450), or 600 mL (600); bags with an oxygen saturated atmosphere (O_2) or compressed air (Air); and transit times of 24 h or 48 h. Letters indicate significant differences ($P < 0.05$) among treatments. No significant difference ($P > 0.05$) was found among treatments within each transit time.

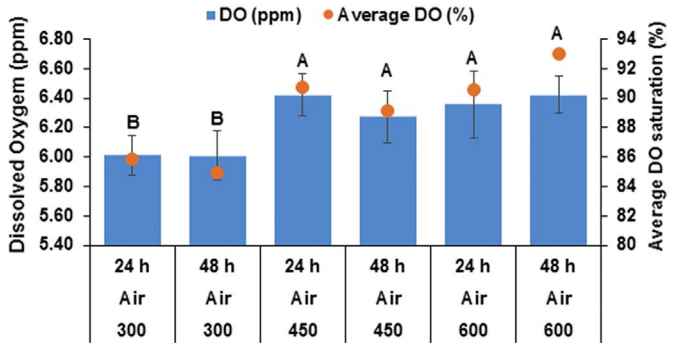

Fig. 6. Concentration of dissolved oxygen in shipping water used to transport live seahorses (*Hippocampus reidi*). Values are averages of five replicates and lines with upper and lower limits represent standard deviations for each treatment. Treatments are: 1 specimen per 300 mL (300), 450 mL (450), or 600 mL (600); bags with compressed air (Air); and transit times of 24 h or 48 h. Letters indicate significant differences ($P < 0.05$) among treatments.

seahorse species occurring in estuaries, such as *H. reidi* and *H. abdominalis* that thrive in salinities as low as 10 (Hora et al., 2016; Martínez-Cárdenas and Purser, 2016).

Treatments performed at a lower density (1 ind. per 600 mL) exhibited a significantly lower decrease in pH when shipped for 48 h, as the accumulation of CO_2 resulting from fish respiration was less likely to promote a drop in pH due to the higher water volume employed per fish biomass. Even at the highest density tested, the average pH recorded after a longer transit time (48 h) was 7.2, value that appears to be within the physiological tolerance range by *H. reidi*. Water pH of 6.4 was recorded when simulating a 12-h transportation of adult *H. abdominalis*, with a mortality of only 1% (Wright et al., 2007). Nonetheless, a decrease in only 0.5 pH units is known to promote a significant behavioural effect on *H. guttulatus* if exposure is prolonged over one month (Faleiro et al., 2015). To date, no study has ever reported seahorse resistance to acute exposures to lower pH values, a feature that is certainly worth investigating (Cohen et al., 2017).

Our data suggest that specimens that were transported for 48 h decreased their breathing frequency and nitrogen metabolism after the first shipping hours. The highest decrease in pH and weight-specific TAN excreted in treatments transported for 48 h may be a result of higher CO_2 and ammonia buildup in shipping water; however, when these values where standardized per hour, the water used to ship seahorses for 48 h showed a significantly lower rate of pH decrease and weight-specific TAN excreted. It is likely that seahorse nitrogen metabolism was more accelerated in the first 24 h due to the handling and packing stress (which consequently increased breathing frequency and ammonia excretion). Only a few hours after being packed, the physiological condition of seahorses may have returned to its basal level. This assumption is supported by the lower decrease of pH values recorded, as well as weight-specific TAN excreted for treatments after 48 h. Curiously, an inverse trend was recorded when transporting clownfish (*Amphiprion ocellaris*), as ammonia excretion rate increased during shipping (Chow et al., 1994). A study addressing *H. abdominalis* revealed that seahorses can rapidly recover from stress caused by packing (Wright et al., 2007). The apparent fast stress-recovery response displayed by seahorses is an important feature from a commercial perspective as live seahorse may be successfully packed in higher densities for longer transit times without negatively affecting their welfare.

Dissolved oxygen concentrations apparently did not affect respiration and nitrogen metabolism. No significant differences were found on

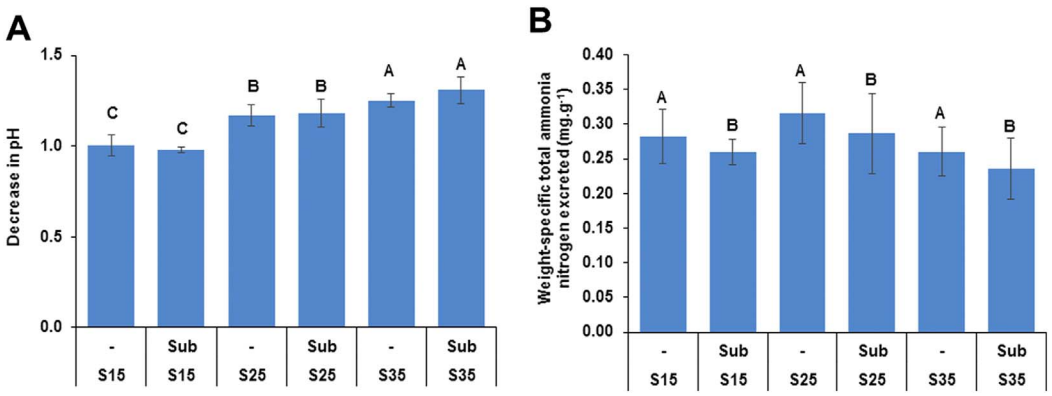

Fig. 7. Decrease in pH (A) and weight-specific total ammonia nitrogen excreted (B) in shipping water used to transport live seahorses (*Hippocampus reidi*). Values are averages of five replicates and lines with upper and lower limits represent standard deviations for each treatment. Treatments are: salinity 15 (S15), 25 (S25), and 35 (S35), with substrate (Sub) and without substrate (-). Letters indicate significant differences ($P < 0.05$) among treatments.

the decrease in pH and weight-specific TAN excreted between treatments employing compressed air or an oxygen saturated atmosphere. The lack of significant effect on the use of an oxygen saturated atmosphere revealed that DO was not a limiting factor under the experimental design essayed. Treatments in higher densities but supplied with compressed air and exposed to a longer transit time (48 h) showed significantly lower DO concentration, but still above 80% of DO saturation. This result showed that seahorse display low oxygen consumption levels, even under potentially stressing conditions caused by transportation.

Lower water salinity may have reduced the breathing frequency of *H. reidi* during transportation. Salinity affected the drop in pH values recorded during shipping, with lower salinity values promoting a lower pH decrease. *Hippocampus reidi* have an isosmotic point of approximately 12 (Hora et al., 2016). When fish are transported at a salinity close to their isosmotic point, they may reduce the energetic burden associated with osmoregulation and, consequently, handle in a more efficient way osmoregulation dysfunctions promoted by shipping stress (Long et al., 1977; McDonald and Milligan, 1997; Weirich and Tomasso, 1991). This rationale has been long used when shipping live freshwater fish, as the addition of salt to the shipping water reduces the difference to the fish isosmotic point (Lim et al., 2007). Marine fish are hyposmotic in relation to their environment, and thus, by reducing the salinity of shipping water they may be able to reduce oxygen demand and improve shipping conditions. Indeed, juveniles of cobia (*Rachycentron canadum*) showed a better performance when transported in water with a salinity 12, likely due to a decrease in their metabolic rate (Stieglitz et al., 2012). Although water in treatments with a salinity of 15 displayed the lowest pH value at the end of the trial (6.95), they experienced the lowest decrease in pH during shipping. This was because the packing water initially displayed a pH of 7.0 due to the need to add freshwater to achieve the desired salinity (15). Overall, our results support the hypothesis that the shipping of seahorses at a salinity close to their isosmotic point can reduce oxygen consumption and consequently the build-up of CO_2 and decrease in pH. Nonetheless, the differences found between the salinities tested were not dramatic and therefore it is legitimate to assume that live *H. reidi* can be successfully transported at a water salinity raging between 15 and 35. Yet, if one considers longer transit times, the differences recorded may not be negligible and a lower water salinity should be preferred.

The present study confirmed that the presence of a substrate, to which seahorses may hold to, may reduce stress and enhance animal welfare during transportation. Seahorses from treatments with substrate excreted significantly less ammonia, which could likely result from a lower stress environment when compared to conspecifics packed without a substrate. Seahorses are benthonic fish that remain long period attached to different substrates, from seaweed, to sponges, corals and mangrove roots (Foster and Vincent, 2004; Kuiter, 2009). It is therefore reasonable to assume that, when being shipped, seahorses that are attached to a substrate will likely be less stressed, especially if one considers the movements to which the Styrofoam box is exposed during transportation. Box movements are inevitable during transportation and fish that are not able to hold to a substrate will have to expend more energy to keep their upright position in the shipping bag. Stressed fish have many physiological imbalances, which may prompt higher ammonia excretion (Randall and Tsui, 2002). Although the weight-specific TAN excreted showed a small difference between treatments with and without substrate, the use of a substrate may promote more contrasting results during longer transit times. Additionally, all seahorses provided with a substrate were holding to it at packing, as well as at when unpacking after transportation. This substrate represented solely an increase of ~5 g (< 1.5%) of the total weight of each shipping unit (bag + seawater). Therefore, the addition of a substrate, to which seahorse may hold to, can enhance welfare and do not represent a significant economic burden in terms of shipping costs.

Considering both experiments performed, the low concentrations of unionized ammonia recorded after transportation, low oxygen consumption and the tolerance to exposure to lower pH it is legitimate to affirm that live seahorses can be successfully shipped over long periods of time in closed plastic bags without significantly affecting their welfare and survival. Nonetheless, temperature and feeding are two important factors that can affect live seahorse shipping success and should be further studied. Both experiments were performed in a room with controlled temperature set at 21 °C, which is within the temperature range recommended for the shipping of tropical fish and is compatible with commercial air shipping freight (Froese, 1998; Lim et al., 2007). Nonetheless, temperature can shift during transit and affect oxygen consumption, gas solubility in water and metabolic rate. Long fasting periods may be also a problem for seahorses in transit over long periods, as these fish have a low digestive ability and hence require constant feeding (Koldewey and Martin-Smith, 2010). In this study, some of the specimens were starved for 72 h (24 h preparation + 48 h transport). Although they were apparently healthy at the end of the experiment, shipping for longer periods could be a problem. Seahorses seem to empty their digestive systems within only a few hours (Corse et al., 2015) and thus, starving for > 24 h before packing is not recommended. In fact, reducing the fasting time prior to packing may in fact enhance seahorse welfare without negatively affecting water quality during transportation.

5. Conclusions

The conditions reported in this study allow traders to pack up to 3

times more live seahorses per shipment and reduce shipping costs by 2/3. Currently, there is no consistence on packing seahorses, with some major traders packing *H. reidi* at densities as low as 1 specimen per L. If one considers a common-size Styrofoam box employed for shipping live fish for the aquarium industry (0.50 m long × 0.35 m wide × 0.30 m high), and take into account the findings of the present study, traders shipping 17 seahorses (1 ind. per L) per box could be shipping up to 58 seahorses (1 ind. per 300 mL) for approximately the same cost. For shorter transit times, traders could further reduce their costs by not using pure oxygen to saturate the bag atmosphere. The welfare of traded specimens can be enhanced for longer transit times by using a lower water salinity and adding a substrate to which seahorse can hold to.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aquaculture.2017.09.024.

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